Volume 232, number 2, 399–404 FEB 05895 May 1988

Improvement in HPLC fractionation of thyroxine-containing thyroglobulin tryptic peptides by prior Accell ion-exchange column chromatography

J. Miguel, M. Asuncion, C. Marin, A. Seguido, L. Lamas and E. Mendez*

Unidad de Endocrinología, Instituto de Investigaciones Biomédicas, CSIC and Facultad de Medicina, Universidad Autónoma de Madrid and **Servicio de Endocrinología, Centro Ramón y Cajal, 28034 Madrid, Spain*

Received 13 February 1988; revised version received 18 March 1988

The isolation and purification of the peptides containing the hormonogenic tyrosyl residues in thyroglobulin is of great interest to the understanding of structure-function relationships in this iodoprotein. This is usually performed in reduced alkylated selectively hydrolyzed thyroglobulin by subsequent HPLC fractionation. However, the main difficulty encountered when trying to isolate these peptides is their disproportion with respect to the total number of possible peptides (14 vs a total of 508). Several HPLC runs with different mobile phases are necessary and each run is accompanied with significant losses of the peptides to be purified. In an attempt to improve the separation of the T4-containing peptides in thyroglobulin tryptic digests from the much more abundant iodotyrosine-containing ones, which are present as contaminants, we have used a very simple and fast step prior to the HPLC fractionation as it is a self-packed ion-exchange column chromatography. This preliminary step results in an improvement in the separation of these peptides and leads to a relative enrichment of the hormonogenic peptides falling in different zones of the HPLC chromatogram, which facilitates their subsequent separation and purification by HPLC.

Thyroglobulin; Hormonogenic peptide; Thyroxine; Thyroid hormone; HPLC fractionation; Ion-exchange chromatography

1. INTRODUCTION

The biosynthesis of the thyroid hormones, thyroxine and triiodothyronine, takes place within the polypeptide chains of thyroglobulin, the prothyroid hormone. Tg is a huge glycoprotein (660 kDa, 19 S) composed of two identical subunits (330 kDa, 12 S). Due to the size of the Tg molecule, the determination of its primary structure by conventional techniques of protein chemistry was practically an unapproachable task. However, advances in DNA-recombinant techniques have made it recently possible [1,2]. The availability of Tg's primary structure has again encouraged studies on structure-function relationships, because it has been shown to have a striking interspecies conservation of its hormonogenic domains [3]. The Tg subunit has 2750 amino acids and a signal peptide with 19 amino acids in front of the N-terminal region. Each subunit has 72 tyrosyl (Tyr) residues which, considering their size, is not a high number but rather average. Of the 144 Tyr residues contained in the Tg molecule, only about 25% are accessible to iodination, and of those 36 are iodinated as mono- and diiodotyrosine (MIT and DIT, respectively), approx. 40% (a maximum of 14 hormonogenic Tyr residues) directly participate in hormone formation. Half of them provide the iodothyronine inner ring ('acceptors') and the other half provide the outer iodothyronine ring ('donors'), leaving behind a dehydroalanine chain to form a maximum of 6–7 hormone residues/mol Tg after the peroxidase-catalyzed

Correspondence address: L. Lamas, Instituto de Investigaciones Biomédicas, CSIC, Facultad de Medicina, UAM, Arzobispo Morcillo, 4, 28029, Madrid, Spain

Abbreviations: MIT, monoiodotyrosine; DIT, diiodotyrosine; T4, thyroxine; T3, triiodothyronine; Tg, thyroglobulin; Tyr, tyrosyl; I*, radioiodine; ME, 2-mercaptoethanol

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

coupling. By reduction-alkylation of Tg from several species, hormone-rich peptides ('hormonogenic peptides') have been isolated [4,5]. These peptides have been fractionated and purified by HPLC [6] after hydrolysis with various selective proteolytic agents (trypsin, CNBr, etc.), sequenced and located within the primary structure of Tg by comparison of the sequence found with that derived from the Tg cDNA [7]. Up to now, four main hormonogenic sites have been identified in rabbit and human Tg corresponding to Tyr residue numbers: 5, 1291, 2555 and 2748 [8]. However, due to the high molecular size of Tg, the purification of these peptides is very laborious since, in order to separate them from the more abundant contaminating peptides, many HPLC runs with various mobile phases are required and the yield is very low. In trying to improve this procedure, in this study, we subjected the tryptic digests, prior to the HPLC fractionation, to Accell self-packed cation (CM)- and anion (QMA)-exchange chromatography, eluting the columns with a volatile solvent [9]. The passage through ion-exchange columns results in the enrichment of T4-containing peptides, greatly facilitating their subsequent fractionation and purification by HPLC.

2. MATERIALS AND METHODS

2.1. *Thyroglobulin*

The iodine poor human goiter Tg was kindly provided by Dr John T. Dunn (University of Virginia, Charlottesville, USA). It was prepared from a long standing non-toxic multinodular goiter by filtration of the 100000 × *g* supernate on a Bio-Gel A-5m column [4]. Its iodine content was 0.09% I, corresponding to approx. 4 atoms of iodine per molecule. The molar concentration of Tg was determined from A_{280nm} measurements using A_{280} = 10.0 cm^{-1} and a molecular mass of 660 kDa.

2.2. *Enzymatic iodination*

The reaction mixture contained per ml: 5 μM Tg, 1.25 × 10^{-4} M iodide labeled with radioiodine (I* from Amersham, SA 1 μCi/μatom), 7.5 μg lactoperoxidase ($A_{412/280}$ = 0.8; Sigma), 60 μmol phosphate buffer, pH 7.0, and an H_2O_2-generating system, formed by 5 mg glucose monohydrate (Merck) and 18.75 mU of glucose oxidase (92.5 U/mg; Boehringer). Incubation was carried out at 37°C for 30 min and the reaction was started with the addition of glucose oxidase and stopped by addition of 25 μl of 10% N_3Na keeping the tubes on ice. 5 μl of each sample were used for separation of the I* bound to Tg from that remaining as free iodide by ascending short term paper chromatography on *n*-butanol/ethanol/1 M ammonia (5:1:2, by vol.) in Whatman 3MM paper strips. The number of atoms of iodine bound to the protein and the number of residues of each iodoamino acid per molecule of Tg was calculated after pronase digestion and paper chromatography as previously described [10,11].

2.3. *Reduction, alkylation and trypsin digestion of in vitro iodinated thyroglobulin*

The Tg samples were reduced at room temperature for 4 h at pH 9.5 with 2-mercaptoethanol (Serva) assuming 100 disulfide groups per mole of Tg and using an ME/disulfide group molar ratio of 100:1. Subsequently, the reduced protein was adjusted to pH 8.0 and alkylated at room temperature for 30 min with acrylonitrile (Merck) using an acrylonitrile/ME molar ratio of 2:1. The reaction was stopped by adding twice as much ME than acrylonitrile used for alkylation. The excess reagents were eliminated by overnight dialysis against 0.02% ammonium bicarbonate (May & Baker). The dialyzed reduced alkylated Tg was then subjected to treatment with TPCK trypsin (Sigma) using a 5% (w/w) solution in 1% ammonium bicarbonate, 0.1% SDS for 16 h at 37°C.

2.4. *Fractionation of tryptic digest from iodinated Tg with Accell ion-exchange columns*

The reduced, carboxymethylated Tg tryptic digests were divided into three aliquots (approx. 1.1 mg each): (i) the control sample subjected directly to HPLC fractionation; (ii) the sample passed through a self-packed Accell CM cation-exchange (carboxymethyl) column (0.8–4 cm); and (iii) the sample passed through a self-packed Accell QMA anion-exchange (quaternary methylammonium) column (0.8–4 cm), both from Waters, according to the scheme in fig.1. The columns were equilibrated with 4.5 ml of 0.05 M ammonium acetate buffer, pH 5.0. The samples were subsequently eluted from the columns with 4.5 ml of 20%, 50%, 80% acetonitrile in 0.05 M ammonium acetate buffer, pH 5.0. Finally the columns were washed with 4.5 ml of 0.4 M NaCl. The eluates were desiccated on a rotavapor and were subsequently subjected to HPLC fractionation (see fig.1).

2.5. *Reverse-phase HPLC fractionation of the control sample and the CM and QMA ion-exchange eluates*

Aliquots from the different pools were dissolved in 5% acetonitrile containing 0.7% ammonium bicarbonate (phase A) and were applied on a C18 μBondapak reverse-phase column using a chromatograph consisting of 2 Waters M6000 A pumps, a 680 automated gradient controller and a Waters 480 variable wavelength absorbance detector. The column was eluted over 110 min with a linear gradient of 5% phase B to 100% phase B (100% acetonitrile). The column was run at room temperature at a flow rate of 0.5 ml/min collecting 0.5 ml fractions. Ultrapure water for high-performance liquid chromatography, generated by a RQ4-coupled to a Milli-Q water purification system (Millipore) was used in the preparation of the mobile phases which were always degassed prior to use. After HPLC fractionation, pronase digestion followed by paper chromatography was performed in most fractions to determine the iodoamino acid distribution and more specifically the T4 contained in them.

2.6. *Presentation of results*

The experiments were performed three times and were reproducible.

3. RESULTS

Table 1 summarizes the iodine atoms bound per molecule of Tg, the [^{125}I]iodoamino acid distribution and the number of residues of each iodoamino acid formed by in vitro enzymatic iodination. The yield of coupling was fairly good since as much as 1.0 T4 and 0.1 T3 residues were formed. As mentioned above, fig.1 summarizes the procedure followed in order to test whether ion-exchange column chromatography, prior to HPLC fractionation of the Tg tryptic digests, enhances the separation and subsequent purification of the T4-containing peptides. Table 2 compares the recovery of ^{125}I-containing peptides, of total T4-containing peptides and their fractionation by HPLC from the Tg tryptic digests in the control sample and in those subjected to self-packed ion-exchange column chromatography prior to HPLC. As expected, this preliminary separative step resulted in a fractionation of the tryptic digest which, as seen in the left column of table 2, is different for the sample applied to the CM column than for the one applied to the QMA column: in the first one, 67.5% of the ^{125}I-labeled material was not retained and 23.4% eluted with 20% acetonitrile, accounting between them for more than 90% of the ^{125}I-tryptic digest applied to the column. In the QMA column, however, 34.9% was not retained and 19.7% eluted with 20% acetonitrile, accounting between them for only 54.6% of the tryptic digest applied. While in the CM column almost nothing eluted with 0.4 M NaCl, in the QMA one, 34.9% eluted with this salt. As shown in the second column of table 2, in the CM column the unretained material contained 38.9% of the total T4 in the digest and, more strikingly, the one eluted with 20% acetonitrile, which, as already mentioned, contained only 23.4% of the ^{125}I-material, had 44.9% of the total T4 in the digest. In other words, it was T4-enriched with respect to the control sample. Again the QMA column worked differently with respect to the T4-containing peptides: the unretained material contained 10.6% of the total T4 and the only T4-enriched eluate was that with 50% acetonitrile which, having only 8.2% of the ^{125}I-material, contained 21.8% of the T4 and the eluate with 0.4 M NaCl which, having 34.9% of the ^{125}I, contained as much as 51.9% of the T4 in the tryptic digest.

Fig.2 shows the HPLC fractionation pattern of both the ^{125}I peptides from the Tg tryptic digests (left ordinate) and the T4 distribution among them expressed as per cent of the total T4 in the digests (right ordinate) for the control and some of the samples which showed T4 enrichment. Fig.2A shows the pattern corresponding to the control sample. Though there is a widespread distribution of the ^{125}I-tryptic peptides, most of the ones containing T4 are separated between fractions 38 and 70 of the HPLC chromatogram. Two main zones are seen: zone 1 (between fractions 37 and 57) and zone 2 (between fractions 58 and 70). According to this arbitrary distribution, it can be seen in table 2 that the T4 in the control sample is almost evenly distributed between these two zones and very little T4 is found elsewhere in the HPLC chromatogram. Fig.2B shows the pattern corresponding to the CM unretained material. It can be seen that, in comparison with the control sample, the late eluting ^{125}I-material has been excluded from this eluate and there is only a single T4 peak which, as also shown in table 2, falls almost completely in zone 1, so that there is an enrichment of the T4-containing peptides separating within zone 1. Fig.2C shows the pattern corresponding to the CM eluate with 20% acetonitrile in which the late eluting material has not been excluded. Again in table 2 it can be seen that more than half of the T4-containing peptides fall in zone 1 but there is

Table 1

Degree of iodination, [^{125}I]iodoamino acid distribution and number of residues formed per molecule by in vitro enzymatic iodination of iodine poor (0.09%) thyroglobulin (for more details see text)

Atoms I/ molecule Tg	[^{125}I]Iodoamino acid distribution				Residues/molecule Tg			
	% DIT	% MIT	% T4	% T3	DIT	MIT	T4	T3
23.3	27.7	39.2	17.3	1.5	3.2	9.1	1.0	0.11

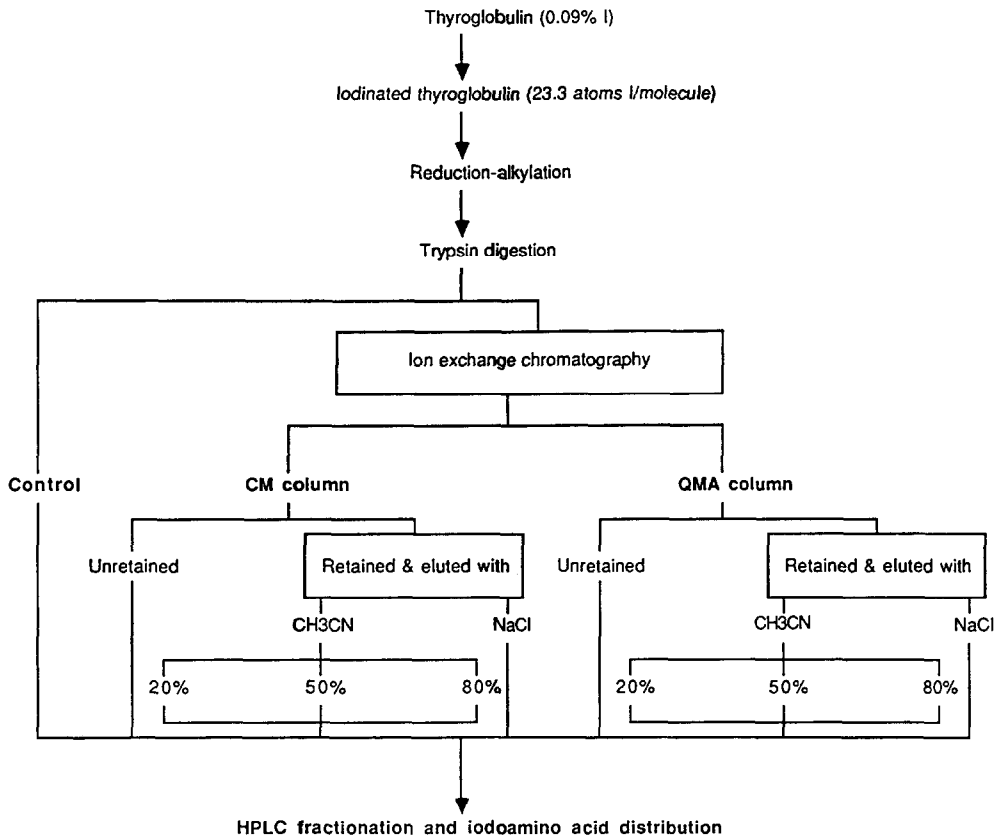

Fig.1. Outline of the procedure used for testing the improvement in separation of T4-containing peptides from thyroglobulin tryptic digests by self-packed ion-exchange column chromatography (for more details see text).

Table 2

Recovery of ^{125}I-containing peptides, of T4-containing peptides and their location in the HPLC chromatogram in Tg tryptic digests after self-packed CM- and QMA-exchange chromatography and elution with various solvents

Sample			% total ^{125}I	HPLC separation of T4-containing peptides			
				% total T4	T4 location		
					Zone 1	Zone 2	Remaining
Control			100	100	54.3	43.5	2.2
CM-exchange column	unretained		67.5	38.9	95.2	–	4.8
	retained eluted with	20% CH_3CN	23.4	44.9	63.1	27.8	9.1
		50% CH_3CN	7.5				
		80% CH_3CN	0.7	16.2	nd	nd	nd
		0.4 M NaCl	0.3				
QMA-exchange column	unretained		34.9	10.6	40.7	–	61.3
	retained eluted with	20% CH_3CN	19.7	15.6	97.6	–	2.4
		50% CH_3CN	8.2	21.8	35.5	59.9	4.6
		80% CH_3CN	2.0	–	nd	nd	nd
		0.4 M NaCl	34.9	51.9	64.4	–	35.6

nd, not determined

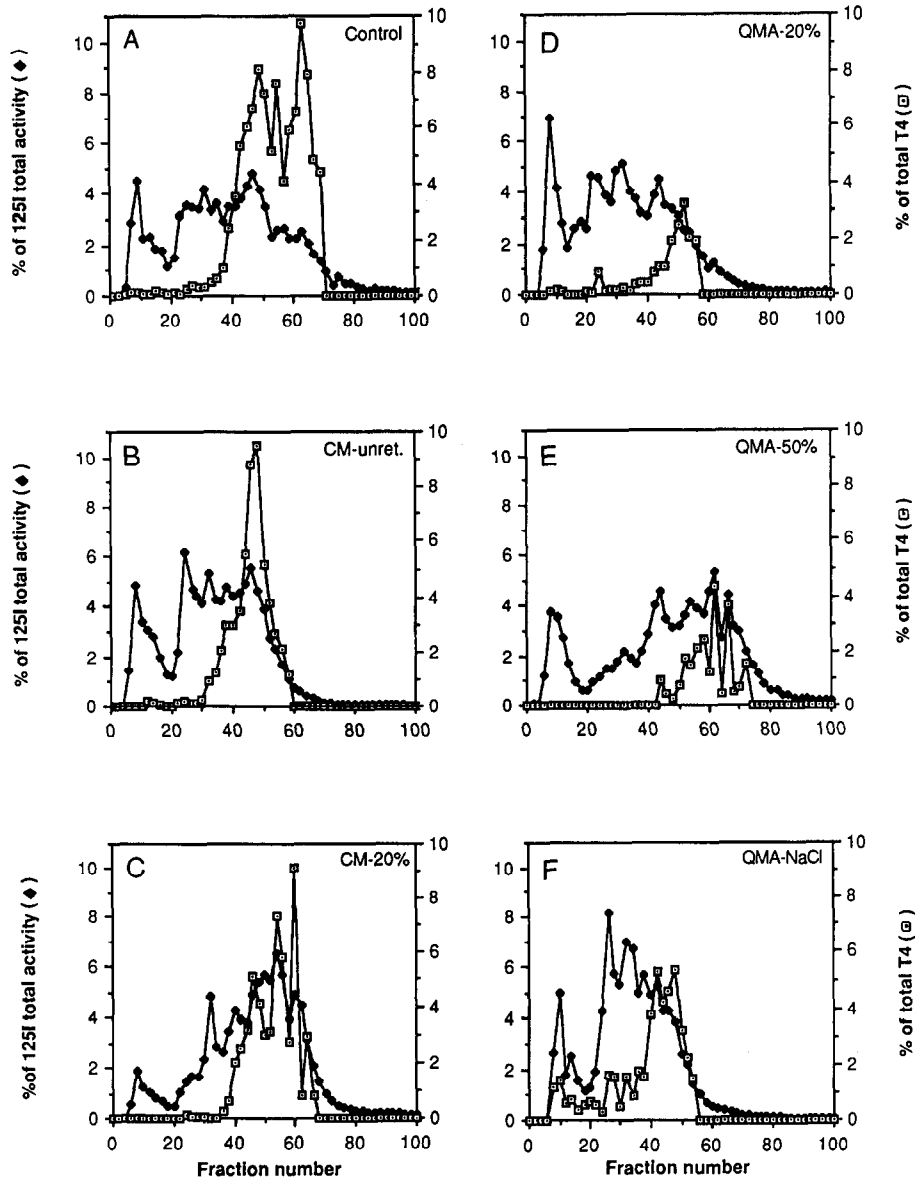

Fig.2. Separation of tryptic peptides of in vitro iodinated (^{125}I) thyroglobulin by reverse-phase HPLC on a C18 μBondapak column. Gradient from 5% to 100% phase B over 110 min. Flow rate 0.5 ml/min. (◆---◆) Percent of total ^{125}I in the chromatogram, (⊡—⊡) T4-containing fractions in the chromatogram after pronase digestion and paper chromatography of aliquots of each fraction, expressed as percent of the total T4 in the control.

also about one fourth which falls now in zone 2. Given the small amount of material eluting in the CM column with 50 and 80% acetonitrile and with 0.4 M NaCl, no attempt was made to fractionate them by HPLC. Fig.2D shows the pattern corresponding to the material eluting with 20% acetonitrile from the QMA column. In this case, the T4-containing peptides fall almost completely in zone 1, as seen also in table 2. Fig.2E shows the pattern corresponding to the QMA 50% acetonitrile eluate which excluded both most of the early and the late eluting material. As can be seen also in table 2, more than half of its T4-containing peptides fall in zone 2. Finally, fig.2F shows the

pattern of the QMA 0.4 M NaCl eluate which almost totally excluded the late eluting material. As can also be seen in table 2 more than half of its T4-containing peptides fall in zone 1.

4. DISCUSSION

Due to the large size of the Tg molecule, the total number of peptides is very high compared with the low number of hormonogenic peptides (in bovine Tg there are 508 possible tryptic peptides but there are only 8–14 directly involved in thyroid hormone formation) and the purification by HPLC fractionation of the latter from the contaminating peptides is very laborious. A high number of HPLC runs is necessary and since each run is accompanied with significant losses of the material to be purified, it is desirable to limit the number of runs as much as possible. In an attempt to improve the separation and subsequent purification of the T4-containing tryptic peptides from the contaminating ones in Tg, we thought that the use of a preliminary simple step, such as a self-packed ion-exchange column chromatography [9], could be useful prior to the HPLC fractionation steps. As shown here, ion-exchange column chromatography of the Tg tryptic digests leads to the separation of various fractions in which not only some enrichment of the overall T4-containing peptides was seen but also a selective enrichment of some of the T4-containing zones. For instance, the cationic unretained material and the 20% acetonitrile eluate together with the anionic 20% acetonitrile and 0.4 M NaCl eluates lead to an enrichment of the T4-containing peptides falling in zone 1, while the anionic 50% eluate leads to an enrichment of the T4-containing peptides falling in zone 2. Though the isolation and purification of the T4-containing peptides in either of the arbitrarily defined zones 1 and 2 was beyond the scope of this paper, according to our experience in separating Tg tryptic peptides by HPLC [12] it is possible that those zones correspond to different hormonogenic peptides. On the other hand, the enrichment of T4-containing peptides was only relative since no complete separation of the two zones was achieved by ion-exchange chromatography. It is possible that connecting or combining the CM and QMA columns [9] could improve the separation and enrichment of the T4-containing peptides. This will have to be tested for each case according to the particular characteristics of the specimen analyzed. We believe that even if, as mentioned above, the enrichment of T4-containing peptides was only relative, the use of ion-exchange chromatography represents an improvement and it can be used, as a preliminary step, when trying to separate and purify not only T4-containing peptides or 'acceptors' but also the iodotyrosine-containing ones acting as 'donors' in the coupling reaction to form the hormones T4 and T3, since it could facilitate their subsequent separation and purification by HPLC.

Acknowledgements: Supported in part by grants from CAYCIT and FIS (Spain) and Comité Conjunto Hispano-Norteamericano no.CCA8411/044.

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